FIRST-GENERATION EFFECTS ON DEVELOPMENT TIME OF OUTCROSSING BETWEEN GEOGRAPHICALLY ISOLATED AND SEASONALLY ISOLATED POPULATIONS OF PINK SALMON (*ONCORHYNCHUS GORBUSCHA*)

By

Jesse D. Echave

RECOMMENDED:

Dr. Milo Adkison

Dr. William Smoker

Dr. Anthony Gharrett, Advisory Committee Chair

Dr. Trent Sutton, Program Head, Fisheries Division

APPROVED

Dr. Michael Castellini,
Interim Dean of the School of Fisheries and Ocean Sciences

Dr. Lawrence Duffy, Interim Dean of the Graduate School

Dec 7, 2010

Date

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A

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Jesse D. Echave, B.S.

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Abstract

Bootstrap analyses of hatch data collected during two independent experiments revealed that hybridization between pink salmon (*Oncorhynchus gorbuscha*) breeding populations separated at either a large geographic scale or a fine temporal scale can influence development time. Restricted maximum likelihood estimators also revealed that sire, dam, cross, and parental interaction can influence genetic variance associated with development time at either scale. Few studies have investigated the extent of local adaptation that results from fine-scale ecological variation, the genetic underpinnings of that adaptation, or the potential impacts outbreeding at that level may have on fitness. We tested whether or not local adaptation contributed to genetic divergence among subpopulations of pink salmon that overlap temporally within the same spawning habitat (early-run fish and late-run fish within Auke Creek, near Juneau, Alaska) by determining whether or not outbreeding influenced development time (a fitness-related trait) in first-generation hybrids. We examined genetic divergence among populations isolated at a much broader scale (Pillar Creek on Kodiak Island, Alaska, and Auke Creek, 1,000 km great circle distance) as a more extreme reference to local adaptation. Results provide evidence that development time is locally adapted and expressed primarily in a locus-by-locus manner.

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Introduction

Widespread distribution and homing behavior in Pacific salmon can reduce gene flow and lead to locally adapted natural populations (Carvalho 1993). Not only can selective pressures among populations differ, but environmental conditions within a single stream often vary spatially and seasonally both within the life span of an individual and between generations. In response to these conditions, natural selection may increase the frequency of "favorable" genes and gene complexes in a salmon population. If reproductive isolation occurs, the frequencies in the populations of genes and gene complexes may diverge as a result of both random drift and local differences in selection regimes until the populations are genetically distinct (reviewed by Taylor 1991; Adkison 1995). Homing behavior increases reproductive isolation and facilitates local adaptation. Most importantly, reproductive isolation, homing, and local adaptation are interrelated, the overall consequence of which is divergence among salmon populations in fitness-related characteristics (Taylor 1991).

From a practical perspective, it is important to realize that genetically distinct populations or spawning segments are not only specifically adapted to a certain environment or series of environments; but they may be poorly adapted to others. Hatchery production and stock transplant, which are used as management tools, can lower natural geneflow barriers. Genetic introgression of hatchery stocks (which may be derived from transplanted fish and may have experienced domestication selection) into

wild stocks may alter the genetic composition responsible for local adaptation and lead to reduced fitness (Hindar et al. 1991).

Just as geneflow barriers can lead to divergence, the removal of geneflow barriers can reduce divergence and, if local selective forces are acting, disrupt local adaptation. When individuals from genetically distinct, locally adapted populations interbreed, the genetic foundation of locally adapted traits may be disrupted; and outbreeding depression (OBD) can result (Lynch 1991; Falconer and Mackay 1996; Lynch and Walsh 1998). Local adaptation and genetic divergence between populations are difficult to quantify, but empirical evidence for whether or not they have taken place can be gained indirectly by observing the effects of experimental outbreeding.

Outbreeding depression may occur in the first generation or may be delayed until the second and later generations depending on whether the genetic foundation for local adaptation is predominately locus-by-locus or involves coadapted gene complexes. If natural selection has operated on many independently acting genes, OBD would likely occur in the first generation (Lynch and Walsh 1998; Gharrett et al. 1999b). If coadapted gene complexes are primarily responsible for increased fitness in a specific environment, OBD will most likely reveal itself in the second or later generations (Emlen 1991; Lynch and Walsh 1998). Under a locus-by-locus model, outbreeding can modify genetic architecture and produce phenotypic intermediates that are unfit in either parental environment (Falconer and Mackay 1996; Lynch and Walsh 1998). Under a coadapted-gene-complex model, OBD occurs when successful gene interactions are disrupted by introgression of the foreign genes into the genomes of recipient populations following

independent assortment and recombination during gametogenesis. Therefore, OBD would not be expected in first-generation hybrids because complete copies of both parental coadapted genomes are present.

Development time influences when salmon fry emerge. Emergence when environmental conditions are optimal for growth and survival has the potential to become a locally adapted trait (Walters et al. 1978; Taylor 1980; Joyce 1986; Goddard 1995; Hebert et al. 1998). In pink salmon, fry migrate directly to sea before they begin to feed. Consequently, emigration timing, yolk absorption, optimal marine temperatures, and estuarine food availability must coincide in order for fry survival to be high (Taylor 1980; Heard 1991). Early salmonid development is plastic in that embryos develop faster at warmer temperatures and slower at cooler temperatures, although the relationship is not absolutely linear (Alderice and Velsen 1978; Heming 1982; Murray and McPhail 1988). Genetic variability among pink salmon populations with respect to development time suggests that each locally adapted population has a unique range of developmental plasticity that relates to local temperature regime, optimum run timing, and competition (redd superimposition) in a way that allows emergence and emigration to occur when the likelihood of survival is favorable (Taylor 1980; Joyce 1986; Goddard 1995; Fukushima et al. 1998; Hebert et al. 1998; Smoker et al. 1998). Non-optimal rates of development can, therefore, reduce survival. For these reasons, development time is pertinent to studies of local adaptation and outbreeding because it offers an opportunity to study readily observable phenotypic differences that have a genetic basis and are directly related to fitness.

Restrictions in gene flow and associated locally adapted traits are easily detected when salmon populations are separated by a large geographic distance for an extended time. The hypothesis that local adaptation contributes to genetic divergence among populations characterized by geographic separation has been tested through the artificial relaxation of natural geneflow barriers (Gharrett and Smoker 1991; Philipp and Claussen 1995; Gharrett et al. 1999a; Gharrett et al. 2001; Gilk et al. 2004; Cooke and Philipp 2006; Wang et al. 2006; Wang et al. 2007). Less understood is the extent of local adaptation and genetic divergence among subpopulations that are in nearby streams or that are seasonally separated within the same stream. Furthermore, little is known about the consequences of relaxing natural geneflow barriers that operate at these levels.

Over the past several decades, local adaptation, inheritance of fitness-related traits, and OBD in naturally occurring pink salmon populations have been studied extensively Auke Creek in Juneau, Alaska. A permanent weir system captures all upstream and downstream migrants, and a fish hatchery allows controlled breeding experiments to be carried out on site with minimal genetic interaction between experimental and wild fish. Research has resolved multiple levels of pink salmon population structure in Auke Creek. Even and odd brood lines in Auke Creek are genetically distinct and second generation interbrood hybrids experienced reduced marine survival (Gharrett and Smoker 1991; Gharrett et al. 1999a). Gilk et al. (2004) described reduced marine survival in both first generation (odd brood line) and second generation (even and odd brood line) hybrids between populations in Auke Creek and Pillar Creek (located on Kodiak Island, Alaska, > 1,000 km away). Differences in development time

between second generation hybrids, controls, and backcrosses in this same geographic experiment also indicated OBD and suggested that both coadapted-gene-complex and locus-by-locus mechanisms contribute to local adaptation (Wang et al. 2007). Populations delineated by such obvious spatial and temporal boundaries exchange little genetic information, have experienced different selection regimes, and are expected to be genetically divergent. Hybrid crosses between such populations are likely candidates for OBD.

Local adaptation may also differentiate subpopulations separated by much shorter distances and/or time frames to the extent that OBD would result from interbreeding. The Auke Creek weir, which is operated by NOAA Alaska Fishery Science Center, is located just above high-tide level at a natural boundary between intertidal and upstream spawners. Fin-clipping experiments revealed that movement of fish between the two areas is not substantial (Taylor 1980), and the introduction of a genetic marker in 1979 and 1981 demonstrated that gene flow between the two groups is limited (Lane et al. 1990; Gharrett et al. 2001).

Auke Creek also experiences bimodal returns of pink salmon each year, with peaks of returning adults occurring in mid to late August and early to mid September (Taylor 2008). Fin-clipping and genetic-marking experiments that showed limited movement of fish or gene flow between intertidal and upstream spawners yielded similar results for early (late August) and late (early September) spawning segments (Lane et al. 1990; McGregor et al. 1998; Smoker et al. 1998; Gharrett et al. 2001). Seasonal changes in water flow and temperature during the time that separates these return groups are

substantial (Taylor 2008). Despite rapid accumulation of temperature units by early-spawned embryos during the warmer weeks of August, a compensatory element in development rate produces a separation of emigration times between early and late fry in the spring that is similar to the separation of immigration time (Gharrett and Smoker 1993; Taylor 2008). Further evidence for reduced gene flow and the potential for local adaptation were observed by studying the heritability of return timing (Smoker et al. 1998) and development time (Joyce 1986; Goddard 1995; Hebert et al. 1998). Return timing and development time could, therefore, influence fitness in either an early or late season temperature regime. Consistent with Wang et al. (2007), Goddard (1995) and Hebert et al. (1998) also suggest that coadapted-gene-complex and locus-by-locus mechanisms for local adaptation in Auke Creek are not mutually exclusive. This evidence suggests another layer of complexity in Auke Creek pink salmon. Experimentally increasing gene flow between early and late spawning segments in Auke Creek in order to observe putative OBD in hybrids offers a unique opportunity to test the hypothesis that local adaptation contributes to genetic divergence among closely allied subpopulations.

In two independent controlled experiments, we compared the effects of removing a large-scale geographic barrier to gene flow (between populations within the same brood line from different geographic regions) with the effects of removing a fine-scale temporal barrier to gene flow (between subpopulations within the same brood line and stream, which exhibited slightly different run times) in order to determine if local adaptation contributes to genetic divergence among spawning groups at both scales. The strict two-

year semelparous life cycle of pink salmon provided an opportunity to replicate each experiment between even and odd brood lines. We investigated the consequences of removing these barriers to gene flow by examining development time in first-generation control and hybrid families. First, we conducted bootstrap analyses to examine differences between control and hybrid families in development time. We then used a restricted maximum likelihood approach to partition variance associated with development time into genetic and environmental components (Lynch and Walsh 1998; Fisher and Bennett 1999) in order to determine which factors (block, cross, timing, dam, sire, or parental interaction) significantly influence the trait. Our observations allowed us to test the hypotheses that 1) OBD occurs in hybrids between pink salmon populations isolated on a broad geographic scale, and 2) OBD occurs in hybrids between pink salmon subpopulations that overlap temporally within the same spawning habitat, which is evidence that 3) local adaptation contributes to genetic divergence both among populations separated at a large geographic scale and among closely allied subpopulations.

Materials and Methods

Source populations and general approach

Pink salmon were taken from two source populations at approximately the same latitude: Auke Creek north of Juneau, Alaska (approximately 58°23'N, 134°37'W), and Pillar Creek on Kodiak Island, Alaska (approximately 57°47'N, 152°28'W). Auke Creek, a high gradient lake-fed stream, is approximately 350 meters long and supports pink salmon breeding populations that have ranged from less than 1,000 to nearly 30,000 fish (Taylor 2008). Pillar Creek is a shallow-gradient, reservoir-fed stream that is approximately 1,800 meters long and supports similar annual returns that range from 1,000 to 40,000 fish (Gilk et al. 2004). Natural gene flow between these two streams is limited by a great circle distance of more than 1,000 km and a continental shelf distance that is considerably larger (Gilk et al. 2004).

In one experiment, referred to as the geographic experiment, removal of a geographic barrier to gene flow was simulated by fertilizing ova from mature Auke Creek females with semen from mature Pillar Creek males. In a second experiment, the temporal experiment, removal of a temporal barrier to gene flow was simulated by crossing the earliest available returning Auke Creek adults with the latest available returning Auke Creek adults. Cryopreserved semen was used to accomplish the temporal hybrid crosses.

The times elapsed between fertilization and the onset of hatching (begin hatch), the midpoint of hatching (mid hatch), and the completion of hatching (end hatch) were used as measures of development time in order to compare the expression of a fitness-related trait between hybrid and control families and to determine if local adaptation and genetic divergence had occurred among breeding groups. Both experiments (geographic and temporal) were replicated across both even and odd brood lines.

Breeding and incubation – geographic experiment

Breeding and incubation of crosses produced from the geographically isolated populations was described by Wang et al. (2007) and took place at Auke Creek Station (ACS). First-generation hybrids were produced in August 1996 and September 1997 with Pillar Creek males and Auke Creek females. Auke Creek males were used to fertilize eggs from those same Auke Creek females to make controls. In order to avoid the confounding effects of using fish from both early and late Auke Creek spawning segments, only late fish were used. Returning male (sire) and female (dam) Auke Creek pink salmon were collected at ACS and held in pens until 60 to 80 fish of each sex were ovulating or spermiating on the same day. Gametes were taken from haphazardly chosen Pillar Creek sires one day before spawning and transported to ACS. On 29 August 1996, 40 Auke Creek females, 40 Auke Creek males, and 40 Pillar Creek males were crossed to produce 80 full-sib hybrid and 80 full-sib control families. Eggs from each of two randomly chosen Auke Creek dams were divided into four lots. Two randomly chosen

Auke Creek sires and two Pillar Creek sires were used to fertilize the four lots of eggs from each dam and to produce controls and hybrids, respectively. The experimental blocks were replicated with different sets of individuals to produce 20 incomplete-factorial arrays within the even brood line. The same methods and mating design were used to produce odd-broodline hybrids and controls on 3 September 1997.

Hybrid and control crosses were incubated in separate vertical FAL™ (Marisource, Milton, Washington) incubator cabinets. Each cabinet housed 16 trays and each tray was subdivided into 10 incubation compartments. Fertilized eggs from each family were divided into two replicate groups of approximately equal size, which were randomly assigned to two cells within the appropriate cabinet. Both cabinets received equal amounts of water from a common source throughout the entire experiment. To prevent fungal outbreaks, eggs were treated twice a week with formaldehyde (1:6000 in static water) for one-hour periods until just prior to hatching. Water temperature was recorded to the nearest 0.1°C at the same time each day until fish were released.

Breeding and incubation – temporal experiment

Breeding and incubation of crosses produced from temporally distinct runs within Auke Creek took place at ACS. First-generation hybrids and controls were produced in 2005 and 2006 from gametes of mature males and females that were collected during the early and late spawning segments in Auke Creek. To represent early and late spawning

segments within each brood line, male and female Auke Creek immigrants were collected on the earliest and latest possible dates each year.

Temporal separation of contributors to the hybrid crosses combined with the lack of an effective egg preservation technique made long- and short-term cryopreservation of semen necessary (e.g., eggs from early females were fertilized with semen from late males of a prior year since late males of the same year had not yet arrived). Semen was collected from haphazardly chosen late sires and cryopreserved on 11 September 2001 and 6 and 9 September 2002. Subsamples of semen collected from early sires that were used to produce early-run control crosses in 2005 and 2006 were also cryopreserved to produce hybrid crosses with late-run females several weeks later. Fresh semen was diluted in an extender solution (ice cold 5.4% glucose, 9% DMSO, 10% fresh egg yolk), sealed in a common plastic drinking straw, frozen on solid CO_2, and stored in liquid N_2 (Wheeler and Thorgaard 1991). When cryopreserved semen was used, straws were placed in 5°C water for 90 seconds, the contents were emptied into appropriate containers of eggs, and an activator (0.9% NaCl, 0.01 M Tris, 0.02 M glycine, 5 mM theophylline; pH 9.0) was added simultaneously. Eggs were bathed in freshly-thawed semen for five minutes to allow fertilization (Wheeler and Thorgaard 1991).

Warm water temperatures and low flow levels in late July and the first half of August 2005 prevented sexual maturation of the earliest Auke Creek pink salmon. The first collection of experimental adults was made on 22 August 2005, after stream temperatures had dropped from a high of 20.0°C on 13 August to 15.9°C. These fish were held in pens so that 60-80 individuals of each sex were ovulating or spermiating on

the same day. On 27 August, the same replicated-block mating scheme that was described for crosses made during the geographic experiment was used to produce 80 full-sib 'early control' and 80 full-sib 'early dam by late sire hybrid' odd-broodline families. Fresh semen from two early Auke Creek sires and cryopreserved semen from two late Auke Creek sires (collected in 2001) were used to fertilize the four lots from each dam. Females from the 2005 late spawning group were captured on 7 September and held until maturity. On 9 September, we fertilized eggs from these females with the remaining cryopreserved semen from the same 2001 males used in August in order to produce 80 half-sib 'late control' families. On the same day, we also fertilized separate lots of eggs from these females with the remaining semen (cryopreserved two weeks earlier) from the same 2005 early males used in August.

Eighty full-sib 'early control' and 80 full-sib 'early dam by late sire hybrid' even-broodline families were produced in 2006 by following the same blocked incomplete-factorial mating scheme that was used in 2005. Experimental adults were randomly collected on 3, 4, and 5 August and crosses were made on 12 August by randomly choosing mature captives. Fresh eggs were fertilized with fresh semen to produce 'early control' families, and fresh eggs were fertilized with cryopreserved semen from 2002 late sires to produce 'early dam by late sire hybrid' families. 'Late control' and 'late dam by early sire hybrid' families produced in 2006 were lost due to infertility of cryopreserved semen from 2006 early males.

Incubation methods used in 2005 and 2006 followed those described by Wang et al. (2007) for crosses produced in the geographic experiment. In order to keep all four

crosses separate, however, four vertical incubator cabinets were required for each brood line.

Development time

The number of accumulated temperature units (ATUs, the sum of daily above-zero water temperatures in degrees Celsius) between fertilization and begin, mid, and end hatch as well as the number of temperature units accumulated during hatching were observed as measures of development time. These indicators of hatching also mark an important development stage in which hatching embryos have the freedom to move away from localized patches of poor water quality in the stream bed. Begin hatch was defined as the day on which the first individual in a cell hatched, mid hatch was the day on which 50% or more of the individuals in a cell hatched, and end hatch was the day on which the last individual in a cell hatched. Total hatch duration was the time difference between end hatch and begin hatch. Before and throughout hatching, each cell was observed at the same time every day. Those observations were recorded as the number of days since fertilization and as ATUs. Begin hatch, mid hatch, end hatch, and total hatch duration data were recorded for the 2005 and 2006 temporal experiments; only mid hatch data were recorded in the 1996 and 1997 geographic experiments.

Data analysis

Differences in development time at each hatch stage and in total hatch duration between hybrid and control crosses were estimated with a bootstrap technique described in Wang et al. (2007). Factorial mating blocks, which consisted of eight full-sib families that were each divided between two cells, were reduced by random sampling to two pairs of full-sib families with one cell per family. The first pair of families consisted of one hybrid and one control family that shared the first dam in the mating block, and the second pair consisted of one hybrid and one control family that shared the second dam in the mating block (each pair constituted a single half-sib family). Differences in development time between the two families of each pair were calculated and the mean and median differences between hybrid and control crosses were calculated over all the blocks. We produced 20,000 of these bootstrapped means and medians per cross. Since hatching benchmarks were quantified from daily ATUs, each dataset contained many identical data points. Because this characteristic most likely distorted mean values, we focused on medians as a measure of central tendency rather than means. The proportion of iterations in which hybrid mean or median values differed from control values in a single direction was determined in order to estimate significance. Each probability level (reported as P_{MC}) refers to the likelihood that the overall mean or median value differed between hybrids and controls in a single direction but not by a certain magnitude (i.e., that development time in hybrids was slower than that of controls or vice versa). This estimation of significance is conservative because a single iteration used only one quarter

of the dataset (Wang et al. 2007). Data collected from early- and late-run crosses in 2005 were analyzed separately.

Since genetic relationships within and among families were known, we were also able to use analysis of variance to partition total phenotypic variance into within- and among-family components and to test the significance of each factor of the experimental design. Variance components were then expressed in terms of covariance between relatives in order to determine the underlying genetic and environmental components responsible for the observed variation (Lynch and Walsh 1998).

We analyzed variance with a restricted maximum likelihood (REML) approach for mixed models in SAS (PROC MIXED; SAS version 9.1, SAS Institute Inc. Cary, North Carolina). Residuals were tested for normality with Shapiro-Wilk and Kolmogorov-Smirnov tests (PROC UNIVARIATE; SAS). Restricted maximum likelihood provides more robust estimates than standard analyses of variance for complex datasets that include both fixed and random effects, that are characterized by deviations from normality, and that are unbalanced. In REML analyses, variances are calculated directly and likelihood is maximized by removing fixed effects from the model and testing them separately (Lynch and Walsh 1998).

A single mating block represented all independent and interactive factors that affect development rate. Examining the variation among such blocks tested the independence and randomness of the experimental design. The linear model that describes all relevant fixed and random effects on development rate in a single brood line (either 1996 or 1997) of the geographic experiment is (Model I):

$$Y_{ijklm} = \mu + B_i + C_{ij} + D_{ik} + S_{ijl} + D_{ik} * S_{ijl} + \varepsilon_{ijklm},$$

where Y_{ijklm} is the dependent variable (ATUs or time required to reach each stage of hatching). The overall population mean is μ, B_i is the random effect of the i^{th} block, C_{ij} is the fixed effect of the j^{th} cross (hybrid or control) within the i^{th} block, D_{ik} is the random effect of the k^{th} dam within the i^{th} block, S_{ijl} is the random effect of the l^{th} sire within the j^{th} cross and i^{th} block, $D_{ik} * S_{ijl}$ is the effect of the parental interaction between the l^{th} sire and k^{th} dam within the j^{th} cross and i^{th} block, and ε_{ijklm} is the residual random error associated with the m^{th} replicate of the l^{th} sire and k^{th} dam within the j^{th} cross and i^{th} block.

The linear model that describes all relevant fixed and random effects on development rate in a single brood line (either 2005 or 2006 to include both August and September fertilization events) of the temporal experiment is (Model II):

$$Y_{ijklmn} = \mu + B_i + T_{ij} + C_{ik} + D_{ijl} + S_{im} + D_{ijl} * S_{im} + \varepsilon_{ijklmn},$$

where Y_{ijklmn} is the dependent variable (ATUs or time required to reach each stage of hatching). The population mean is μ, B_i is the random effect of the i^{th} block, T_{ij} is the fixed effect of the j^{th} fertilization event (August or September) within the i^{th} block, C_{ik} is the fixed effect of the k^{th} cross (hybrid or control) within the i^{th} block, D_{ijl} is the random effect of the l^{th} dam from the j^{th} spawning season within the i^{th} block, S_{im} is the random

effect of the m^{th} sire within the i^{th} block, $D_{ijl} * S_{im}$ is the effect of the interaction between the m^{th} sire and l^{th} dam (parental interaction) of the k^{th} cross within the j^{th} spawning season and i^{th} block, and ε_{ijklmn} is the residual random error associated with the n^{th} replicate from the mating between the m^{th} sire and l^{th} dam of the k^{th} cross within the j^{th} spawning season and i^{th} block.

In the temporal experiment, different females were nested within each fertilization event (either August or September) during both brood lines; and each event represented a unique thermal regime in which embryos were incubated. Consequently, the effects of dam and fertilization event were confounded. Therefore, development data from each brood line of the temporal experiment were also subdivided into August and September events and analyzed separately by using the model described for the geographic experiment (Model I).

It was assumed that individuals that were used in each experiment were randomly chosen from their populations. Terms that represent dam, sire, and parental effects in both experiments are, therefore, presented as independent random variables with expectations equal to zero. To identify the underlying genetic and environmental contributions to observed phenotypic variation, relevant within- and among-family variance components were expressed in terms of genetic covariance between relatives (Lynch and Walsh 1998). Briefly, in these models, dam variance (σ_D^2) is equivalent to the covariance of maternal half-siblings (COV_{MHS}) and represents one quarter of the total additive genetic variance (V_A) plus maternal effects (V_M) and smaller fractions of epistatic variance. The sire variance (σ_S^2) is equivalent to the covariance of paternal

half-siblings (COV_{PHS}) and represents one quarter of the total additive genetic variance (V_A) plus smaller fractions of epistatic variance. The parental interaction (σ^2_{DS}) is equivalent to the covariance of full-siblings (COV_{FS}) less the maternal and paternal half-sib covariance and represents one quarter of the total dominance (V_D).

Results

Geographic experiment

Differences in development time between geographic hybrid and control crosses were calculated by Wang et al. (2007). Bootstrap analyses revealed that first-generation hybrids between Auke Creek and Pillar Creek developed more slowly than controls from Auke Creek when both crosses were incubated at ACS. Hybrids required approximately 20 additional ATUs to reach median mid hatch than controls in both brood lines ($P_{MC} << 0.001$; Table 1 and Fig. 1; Wang et al. 2007). As predicted by the compensatory element in salmonid development time (Joyce 1986), both hybrid and control crosses from the warmer year of 1997 required more ATUs to reach mid hatch.

Results of REML procedures for the geographic experiment were consistent with a locus-by-locus model for OBD. Additive genetic variance was observed in the form of significant cross, dam, and sire effects in both brood lines. Parental interaction did not influence development time in either brood line when measured at a 5% significance level. However, at a 10% level, parental interaction was significant, which indicates

Table 1. Bootstrap analyses of differences between hybrid and control pink salmon in development time. Each difference was calculated by subtracting the accumulated temperature unit value for controls from the respective value for hybrids and is followed by its Monte Carlo probability value (P_{MC}).

Fertilization date	Hatch stage	Difference between means (P_{MC})	Difference between medians (P_{MC})
Geographic experiment			
29 Aug 1996[a]	Mid	+17.2 (<<0.001)	+20.4 (<<0.001)
3 Sep 1997[a]	Mid	+17.7 (<<0.001)	+19.0 (<<0.001)
Temporal experiment			
27 Aug 2005	Begin	+8.0 (0.004)	+10.1 (0.036)
	Mid	+7.8 (<<0.001)	+3.6 (0.456)
	End	+5.1 (<<0.001)	+6.9 (<0.001)
	Total duration	-2.8 (0.195)	-6.7 (0.085)
9 Sep 2005	Begin	+3.3 (0.001)	+3.7 (0.062)
	Mid	+1.9 (<0.001)	+0.3 (0.869)
	End	0 (NA)	-0.8 (0.706)
	Total duration	-3.3 (0.017)	-1.7 (0.186)
12 Aug 2006	Begin	+0.1 (0.486)	-2.5 (0.501)
	Mid	-3.3 (0.002)	-3.2 (0.340)
	End	-8.0 (<<0.001)	-9.4 (0.006)
	Total duration	-8.1 (0.005)	-8.0 (0.011)

[a] from Wang et al. (2007)

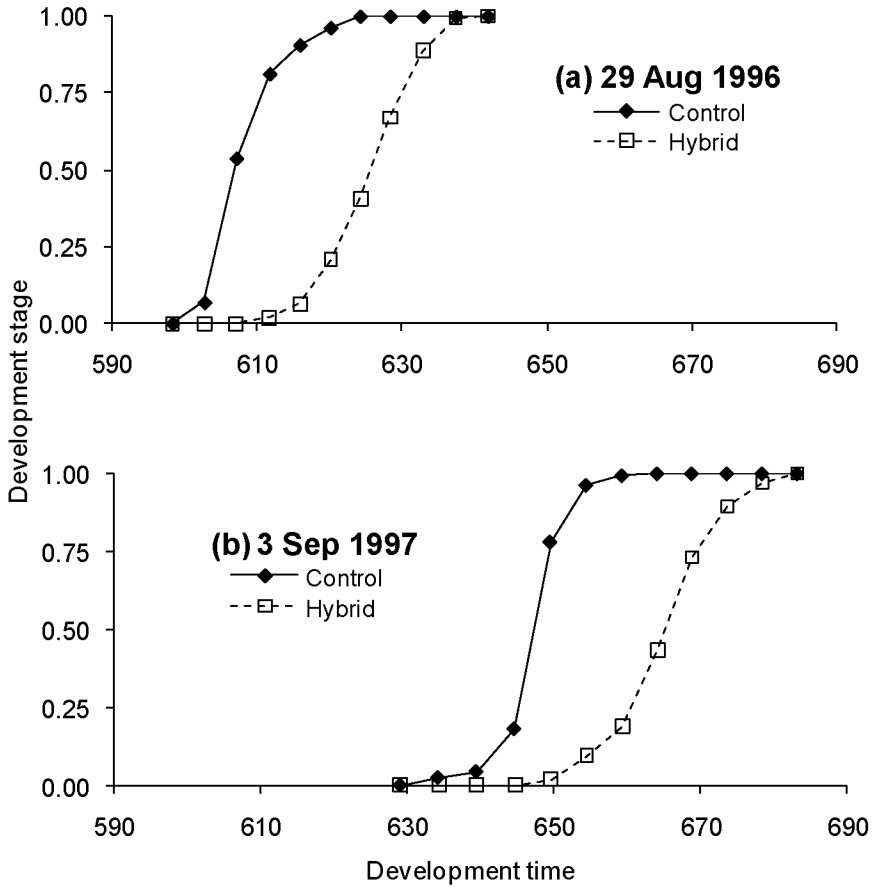

Figure 1. Development time in geographic hybrid and geographic control pink salmon in 1996 (a) and 1997 (b), measured by accumulated temperature units at which half the embryos in each family hatched (Wang et al. 2007).

there was a small non-additive source of variation. Lack of a significant block effect in either brood line was evidence that returning adults collected for the experiment were successfully chosen at random (Table 2).

Temporal experiment – bootstrap analysis

First-generation hybrids between early dams and late sires developed more slowly in the early-run temperature environment of 2005 than did early-run controls that were produced on the same day (Fig. 2a). Approximately 10.1 additional ATUs were required for hybrids to reach median begin hatch, and 6.9 additional ATUs were required to reach median end hatch ($P_{MC} << 0.05$; Table 1). All embryos had hatched by 31 Oct 2005 (Fig. 3).

In contrast to fertilization of early 2005 crosses, fertilization of early 2006 crosses occurred much earlier in the year (Figs. 2b and 3). First-generation hybrids between early dams and late sires developed faster in the early-run temperature environment in 2006 than did early-run controls that were produced on the same day (Fig. 2b). These 2006 hybrids also required approximately 9.4 fewer ATUs to reach median end hatch, and median total hatch duration was 8.0 ATUs shorter for hybrids than controls ($P_{MC} << 0.05$; Table 1). The last embryo did not hatch until 17 November 2006 (Figs. 2b and 3). The average daily water temperature from fertilization to end hatch for these crosses was 6.7°C, which was 4.1°C cooler than the incubation temperature for early crosses in 2005 (Fig. 3). This lower average temperature accounted for an increased number of days

Table 2. Factors that influenced development time in geographic hybrid and geographic control pink salmon in 1996 and 1997. Significance values are given for block (B), cross (C), dam (D), sire (S), and parental interaction (D*S) effects. Analyses were conducted with restricted maximum likelihood (REML) for mixed models (PROC MIXED) in SAS (version 9.1). Significance values of $p < 0.05$ are marked in bold.

Brood (model)	Source	Contribution of each source of genetic variation to development time (p value)
29 Aug 1996 (model I)	Block (B)	0.192
	Cross (C)[a]	**<0.001**
	Dam (D)	**0.012**
	Sire (S)	**<0.001**
	D*S	0.083
3 Sep 1997 (model I)	Block (B)	0.132
	Cross (C)[a]	**<0.001**
	Dam (D)	**0.008**
	Sire (S)	**<0.001**
	D*S	0.065

[a]Fixed effects. All other effects are random.

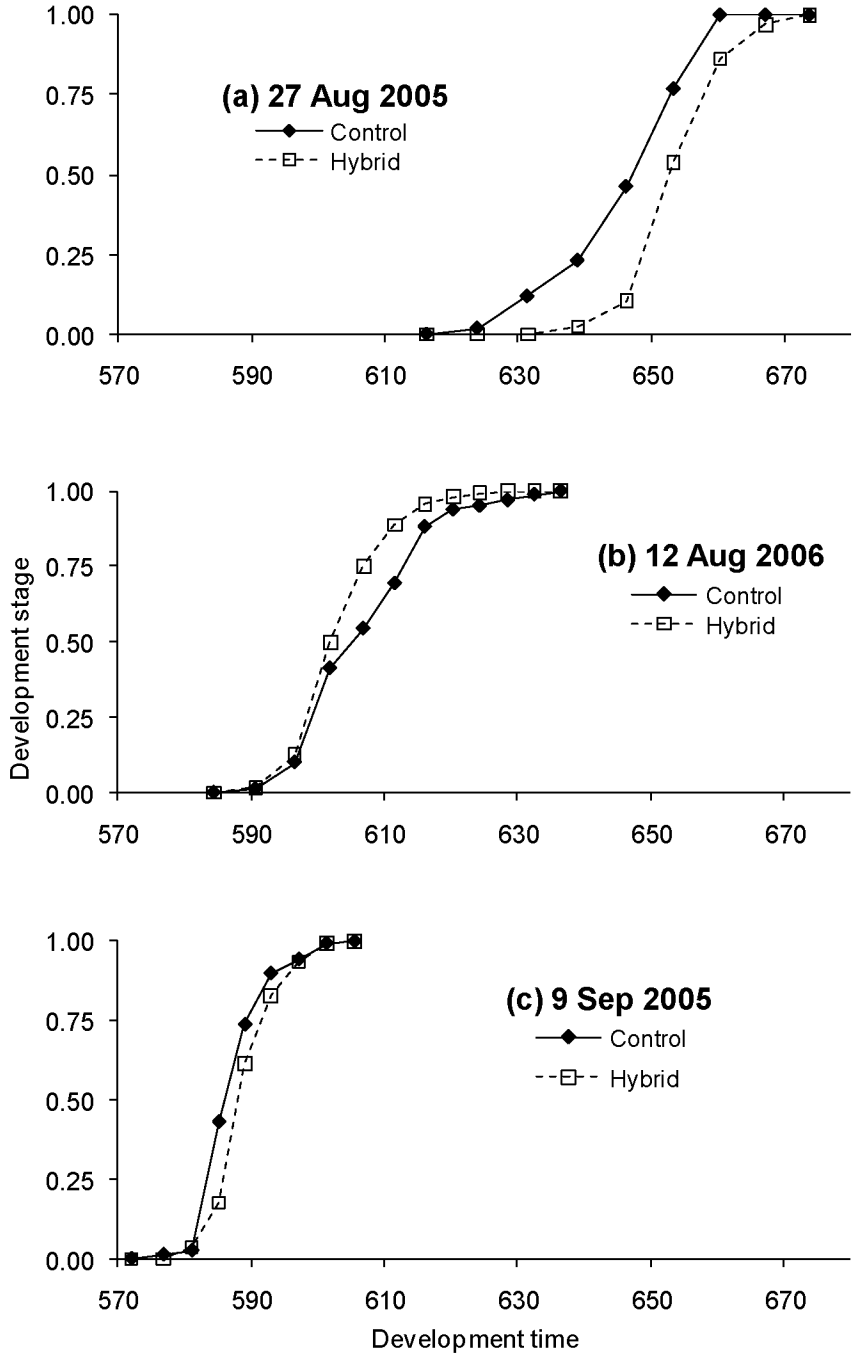

Figure 2. Development time in temporal hybrid and temporal control pink salmon in August 2005 (a), August 2006 (b), and September 2005 (c), measured by accumulated temperature units at which half the embryos in each family hatched

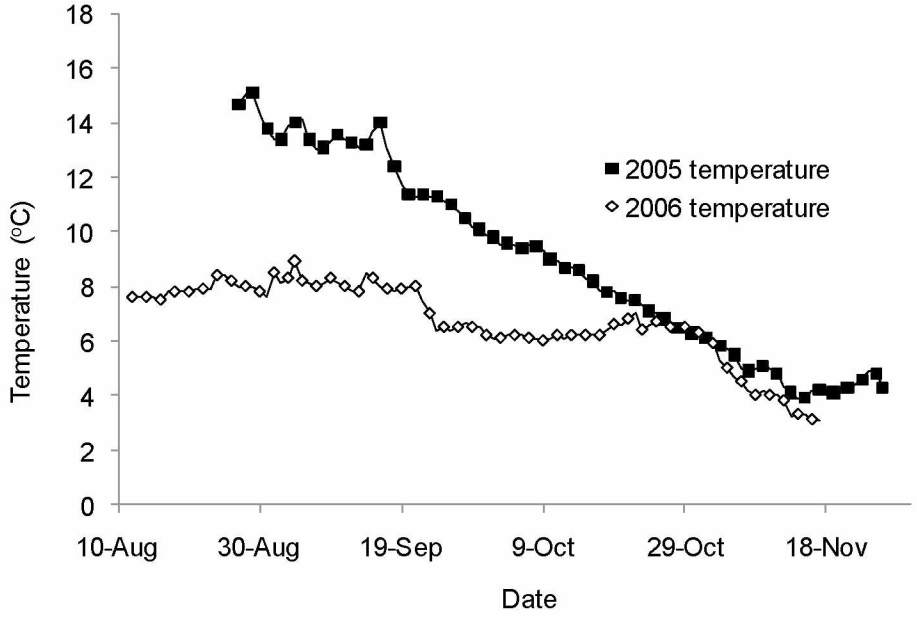

Figure 3. Daily incubation temperatures for the temporal experiment. Temperature data are depicted for the time period between when the first and last embryos hatched each year. Average incubation temperatures for early-run 2005 crosses, early-run 2006 crosses, and late-run 2005 crosses were 10.6°C, 6.7°C, and 8.1°C, respectively

between fertilization and end hatch when compared to early crosses produced in 2005, but fewer overall ATUs. Results for means from the two early runs paralleled those of medians (Table 1).

In the late-run temperature environment of 2005, first-generation hybrids between late dams and early sires did not generally differ in development time from late-run controls ($P_{MC} > 0.05$). However, the difference of 3.7 ATUs at begin hatch exceeded zero at $0.05 < P_{MC} < 0.1$ (Table 1). All embryos had hatched by 26 November 2005 (Figs. 2c and 3). Again, the results of analyses of means were similar to analyses of medians.

Temporal experiment – REML analysis

In order to determine which factors (block, cross, timing, dam, sire, parental interaction) influenced development time, we analyzed variance associated with each hatch dataset (from either August 2005, August 2006, or September 2005 fertilization events) separately with model I; and we analyzed variance associated with the entire 2005 data set (both August and September) with model II. Cross significantly influenced development time when measured at begin and mid hatch in both August and September temperature environments as well as development time measured at end hatch in August and total hatch duration in September ($p < 0.05$). Sire and dam significantly influenced development time measured at mid and end hatch in both August and September

temperature environments and dam influenced total hatch duration in September ($p < 0.05$). Block effects were not significant (Table 3).

When the entire 2005 dataset was collectively analyzed (model II), we observed highly significant cross, and dam/timing (temperature environments resulting from different fertilization events) effects for every hatch stage as well as for total hatch duration. Accumulated temperature units to mid and end hatch under model II were also highly influenced by sire ($p << 0.05$). Again, the design does not partition fertilization events from dams, so the source of parental interaction is equivocal under Model II. Block effects were not significant (Table 3). These results support a locus-by-locus model for OBD.

Results of REML for August 2006 crosses were also consistent with a locus-by-locus model for OBD. Time to mid hatch was significantly influenced by cross, dam, and sire. Time to end hatch and total hatch duration were significantly influenced by cross ($p < 0.05$). Again, block effects were not significant (Table 3).

The REML procedure revealed a small non-additive influence (V_D) in the geographic experiment. Parental interaction influenced time to end hatch ($p = 0.037$) under Model II for 2005, and time to begin hatch in August 2006 ($p = 0.037$). However, measurement of begin and end hatch depended on single individuals and therefore may not be as representative of each family as mid hatch. In contrast, parental interaction had no significant influence on mid hatch throughout the temporal experiment (Table 3).

Table 3. Factors that influenced development time in temporal hybrid and temporal control pink salmon in 2005 and 2006. Significance values are given for block (B), cross (C), timing (T), dam (D), sire (S), and parental interaction (D*S) effects. Analyses were conducted with restricted maximum likelihood (REML) for mixed models (PROC MIXED) in SAS (version 9.1). Significance values of $p < 0.05$ are marked in bold.

Fertilization date (model)	Source of genetic variation	Contribution of each source of genetic variation to development time (p value)			
		Begin Hatch	Mid Hatch	End Hatch	Total duration
27 Aug 2005 (model I)	Block (B)	0.093	0.264	0.509	0.372
	Cross (C)[a]	**<0.001**	**<0.001**	**<0.001**	0.138
	Dam (D)	0.380	**0.011**	**0.020**	0.557
	Sire (S)	0.596	**0.002**	**0.001**	0.785
	D*S	0.472	0.831	0.699	0.827
12 Aug 2006 (model I)	Block (B)	0.893	0.627	0.75	0.520
	Cross (C)[a]	0.958	**0.004**	**<0.001**	**0.002**
	Dam (D)	0.161	**0.014**	0.216	0.925
	Sire (S)	0.207	**0.004**	0.727	0.423
	D*S	**0.022**	0.145	0.277	0.056
9 Sep 2005 (model I)	Block (B)	0.872	0.627	0.841	0.475
	Cross (C)[a]	**0.001**	**0.026**	0.989	**0.004**
	Dam (D)	0.123	**0.025**	**0.020**	**0.036**
	Sire (S)	0.122	**<0.001**	**<0.001**	0.109
	D*S	0.230	0.322	0.627	0.683
Aug and Sep 2005 (model II)	Block (B)	0.244	0.757	0.686	0.643
	Cross (C)[a]	**<0.001**	**<0.001**	**<0.001**	**0.005**
	Timing (T)[a]	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	Dam (D)	**0.007**	**<0.001**	**<0.001**	**0.019**
	Sire (S)	0.373	**<0.001**	**<0.001**	0.969
	D*S	0.423	0.439	**0.037**	0.616

[a]Fixed effects. All other effects are random

Discussion

Evidence of local adaptation in the geographic experiment

Auke Creek and Pillar Creek pink salmon represent populations that are geographically separated at a broad scale. First-generation development data for the geographic experiment were collected during a sequence of Pillar Creek and Auke Creek breeding experiments that were carried out to examine the extent of local adaptation in geographically isolated pink salmon populations by determining the potential for that adaptation to cause OBD in hybrids (Gilk et al. 2004; Wang et al. 2007). Prior to the results presented here, analyses maximized the chances of detecting a potential OBD effect by employing tests of high power that focused on areas where a potential breakdown in hybrid fitness would be most obvious. Because the focus was typically on second generation offspring, those tests could also point toward the possible genetic mechanisms involved. First, Gilk et al. (2004) confirmed through microsatellite analyses that Auke Creek and Pillar Creek pink salmon populations differ genetically. Also, there was reduced marine survival in both F_1 (odd brood line) and F_2 (even and odd brood lines) hybrids (Gilk et al. 2004) as well as differences in development time between F_2 hybrids, controls, and backcrosses (Wang et al. 2007), which suggested that both coadapted-gene-complex and locus-by-locus mechanisms contribute to local adaptation.

We further analyzed mid hatch data that were collected from first-generation Auke Creek and Pillar Creek hybrids and controls to continue testing the hypothesis that

local adaptation has contributed to genetic divergence between the two populations. Detection of OBD in first-generation geographic hybrids of both brood lines confirmed results of previous research, suggesting that development time is locally adapted and that genetic divergence between these geographic isolates is due in part to such adaptation. Results of the quantitative genetic analysis, in particular, revealed signs of both additive and dominance variance associated with the expression of development time.

Bootstrap analyses showed significant differences ($p << 0.001$) between when hybrids reached mid hatch and when controls reached the same developmental benchmark (Wang et al. 2007). Since this experiment did not include incubation of Pillar Creek eggs at Auke Creek and vice versa, it is not possible to know whether hybrid mid hatch times were intermediate between the two parent populations, as would be expected under a classic locus-by-locus model. However, observation of OBD effects in the first generation is itself evidence of local adaptation in a locus-by-locus manner and, more importantly, is straightforward empirical evidence that selection associated with development time has contributed to genetic divergence between Auke Creek and Pillar Creek pink salmon populations.

Results of REML analyses confirmed interpretations from the bootstrap analyses. We observed that cross, dam, and sire all influenced development time at mid hatch in first-generation progeny in both brood lines. A significant cross effect demonstrated that development time is unique to a cross and determined by the proportion of Pillar Creek and Auke Creek genetic material present. These results suggested that each spawning segment is uniquely adapted to the temperature profiles of either Pillar or Auke Creek.

Genotype-by-environment interaction and maternal effects associated with dam are difficult to tease out with limited, first-generation data. However, along with sire and cross effects, a significant dam effect is also consistent with a first-generation, locus-by-locus model. Weak parental-interaction (V_D) in the geographic experiment is indicative of non-additive genetic effects. However, this result could simply be due to dominance effects at individual loci which could have resulted from contributions of genetically divergent, spatially separated populations. Strong evidence for disruption of the coadapted genome cannot be obtained from the first-generation data used here, but they might be expected at later generations based on the high degree of geographic and genetic isolation between these two populations.

Evidence of local adaptation in the temporal experiment

It is reasonable that early and late runs of pink salmon in Auke Creek represent locally adapted spawning segments that are seasonally separated within the same brood line and stream. However, previous research in Auke Creek did not explore the consequences of relaxing natural geneflow barriers that operate at these levels. Results of our experiments led us to conclude that local adaptation can contribute to genetic divergence among spawning groups even when subpopulation structure is so fine that it is difficult to discern without extensive or quantitative genetic analysis.

Previous research showed that embryos derived from late-run Auke Creek pink salmon developed more slowly than those derived from the early run, regardless of

incubation regime (early/warm or late/cool; Hebert et al. 1998). Since estuarine productivity is strongly seasonal, optimal emigration time is similar for fry from both runs. On average, early-run fry emigrate roughly two weeks before late-run fry, which is similar to the span that separates spawning in the two subopulations. However, water temperatures in Auke Creek are colder during spring emigration than fall immigration and do not vary substantially, so it is evident that temperature-dependent development times differ between the two subpopulations, a difference that is inherited (Hebert et al. 1998). Therefore, the overall slower development time of early-run fish delays emigration until favorable marine conditions exist, which synchronizes emigration of fish from the two subpopulations with the vernal pulse of productivity in Auke Bay. With this empirical evidence in mind, it was expected that outbreeding between early- and late-run fish would produce offspring throughout the temporal experiment that were intermediate between the two runs in development time. Contrary to this expectation, hybrids between early dams and late sires that were incubated in an early 2005 temperature environment developed more slowly than early-run controls incubated in the same environment.

Incubation temperatures in 2005 were among the warmest on record and were likely related to the unexpected observations (data on file, U.S. NOAA Fisheries, Alaska Fisheries Science Center, Auke Bay Laboratory, Juneau, Alaska 99801). Stream temperatures approached lethal levels for pink salmon embryos (Beacham and Murray 1990), and it is possible that these extreme temperatures were simply outside the range in which homeostatic mechanisms related to development time can successfully act. Hebert

et al. (1998) also showed that despite a faster development time when measured at mid hatch, late-run embryos developed more slowly than early-run embryos until reaching the earlier stages of epiboly and eye pigmentation. Redd superimposition is a major source of mortality of early stage Auke Creek pink salmon embryos, more important than other sources of mortality such as streambed scouring during high stream flow (Fukushima et al. 1998). By reaching epiboly quickly, early-run embryos become resistant to the mechanical shock caused by redd superimposition when late-run fish spawn (Joyce 1986; Hebert et al. 1998). This adaptive early rapid development would increase the risk of premature outmigration of early fish if it was not compensated for by slowing later development, especially if water temperatures are high (Hebert et al. 1998). The average daily water temperature from fertilization to the end of hatching for early 2005 crosses was 10.6°C, which is much warmer than any regime analyzed by Hebert et al. (1998). Therefore, tremendous plasticity was necessary to sufficiently slow development of early-run embryos and prevent premature emigration.

Development time of hybrids between early dams and late sires that were incubated in an early 2006 temperature environment was closer to that predicted by the Hebert et al. (1998) results. Unlike corresponding early crosses produced in 2005, development of hybrids was faster than that of the controls. Because temperatures were closer to normal in 2006, it is likely that results from those crosses provide a better characterization of the intrinsic developmental mechanisms of Auke Creek pink salmon.

Hybrids between late dams and early sires that were incubated in a late regime in 2005 did not differ from late-run controls at a 5% level. At a 10% level, however,

hybrids required more ATUs to reach begin hatch than controls. Average daily temperature during this incubation period was cooler than that for the 2005 early run at 8.5°C, but still relatively warm at the beginning of incubation. Since late dams were used for both hybrid and control crosses, this may also emphasize the importance of maternal effects in that early dams and late dams and may not influence development time to the same extent.

While results of bootstrap analyses were ambiguous, results of REML analyses were far more conclusive. With REML, we directly determined whether or not development time expression had a genetic component, which specific genetic sources among those analyzed contributed to observed variance, and what the magnitude of each contribution was relative to that from other sources. The presence of a genetic effect under our experimental design is unequivocal.

Results of all REML analyses supported a locus-by-locus model for OBD as well as corroborated previous Auke Creek research. Under Model I, cross, dam, and sire significantly affected time to mid hatch in both the early and late experiments in 2005, and in the early experiment in 2006. In addition to timing, these effects were also observed under model II. However, since dam and timing effects are confounded under model II, our focus is on model I. Similar results were observed at other hatch stages, although the pattern was not as consistent. Since the definition of begin hatch, end hatch, and, therefore, total duration of hatching depended on single individuals in a cell, these observations may not be completely representative of each population.

These results were obtained in the first generation, and temporally separated spawning segments are genetically, geographically, and temporally more proximate than any two spawning groups used in the past. Consequently, the quantitative genetic influences of cross, sire, and dam provide strong evidence for additive local adaptation and OBD. These effects were also observed in the geographic experiment and our interpretation of each is similar in both outbreeding scenarios. A significant cross effect in the temporal scenario demonstrates that each development rate measured, and therefore the trajectory mapped by any change of that development rate over time, is unique to a cross and determined by the proportion of early- and late-run genetic material present. The implication is that each subpopulation used in the temporal experiment is uniquely adapted to the temperature cues of either an early- or late-season incubation regime and possibly the mechanical influences of late spawners. Again, genotype-by-environment interaction and maternal effects associated with dam are difficult to separate. However, along with sire and cross effects, a significant dam effect is also consistent with a first-generation, locus-by-locus model for adaptation and OBD. A significant sire effect, in particular, also indicates that the development-time traits examined here have a genetic component. First-generation results such as these are indirect evidence of the heritability of fitness-related development traits in Auke Creek pink salmon. Not only do sire and dam effects in the first generation indicate locus-by-locus genetic variation, they suggest that these traits have been responsive to selection pressures.

While it is clear that non-additive (V_D) genetic effects did not significantly influence the variance associated with development time in the temporal experiment, they may have played a role in geographic crosses. However, these influences are weak at best in a first-generation experiment.

The maintenance of population structure such as that observed within Auke Creek is just as important to fitness as the maintenance of structure observed at much broader scales. In a study of the long-term series of biological and environmental data that exist for Auke Creek, Taylor (2008) observed that air and sea surface temperatures in Auke Bay, total precipitation, and average water temperatures in Auke Creek during periods of pink salmon incubation are all rising. At the same time, the trend is towards an earlier timing of upstream migration of Auke Creek pink salmon. Hebert et al. (1998) emphasized that a high level of genetic diversity is necessary to cope with high temperatures such as those recently encountered in late July and August, which implies that a continuing trend of earlier upstream migration could threaten Auke Creek pink salmon. It has also been noted that the occurrence of adult pink salmon in early July and late September is becoming rare (data on file, U.S. NOAA Fisheries, Alaska Fisheries Science Center, Auke Bay Laboratory, Juneau, Alaska 99801). This shorter spawning period has likely reduced the genetic diversity in Auke Creek relative to that existing 30 years ago when the spawning season extended from early August to October. Ultimately, the combination of earlier upstream migration and warmer environmental conditions has resulted in an earlier emigration of fry in the spring (Taylor 2008). Taylor (2008) also noted that along with warmer surface temperatures and lower surface salinities,

photoperiod is a major driving force for the spring phytoplankton bloom, and therefore increased zooplankton density, in high-latitude estuarine environments like Auke Bay (Ziemann et al. 1990). Timing of the spring phytoplankton bloom and subsequent increases in forage for pink salmon fry typically begins in early April as surface temperatures increase, and due to their relationship with incident light is expected to remain relatively constant (Ziemann et al. 1990). Through development-rate compensation and the continual process of local adaptation, it is probable that populations of wild salmon can adjust to some changes in environmental conditions and that emigration and/or spawning time can shift in a direction that would maintain survival. However, changes or breakdowns of geneflow barriers as a consequence of climate changes can impede this process, especially if environmental conditions are changing rapidly and especially if it occurs in conjunction with a decrease in total genetic diversity.

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